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Hemorrhagic Shock in Swine: Nitric Oxide and Potassium Sensitive Adenosine Triphosphate Channel Activation

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Background: **To determine the role of nitric oxide and adenosine triphosphate–sensitive potassium (K_{ATP}) vascular channels in vascular decompensation during controlled hemorrhagic shock in swine.**

Methods: **Thirty instrumented, anesthetized adolescent Yorkshire swine were subjected to controlled isobaric hemorrhage to a mean arterial pressure of 40 mmHg for 2 h (n = 6) or 4 h (n = 10) or 50 mmHg for 4 h (n = 8). An additional six animals were used as anesthetized instrumented time controls. During controlled hemorrhage, plasma and tissue samples were obtained every 30 to 60 min. Before euthanasia, tissue (carotid artery, lung, liver, and aorta) was obtained for analysis of nitrate concentrations and nitric oxide synthase activity. Isolated carotid artery ring reactivity to norepinephrine was also determined with and without glibenclamide.**

Results: **Animals hemorrhaged to 40 mmHg decompensated earlier than animals hemorrhaged to 50 mmHg. Plasma nitrate concentrations and nitric oxide synthase activity rose consistently throughout hemorrhage in both groups. However, they were substantially higher in the mean arterial pressure 40 group. Constitutive nitric oxide synthase activity was the major contributor to total nitric oxide synthase activity throughout the protocol with only the animals maintained at 40 mmHg for 4 h showing evidence of inducible nitric oxide synthase activity. Profound K_{ATP} channel activation and hyporeactivity of isolated vessel rings to norepinephrine was not observed until 4 h after the initiation of hemorrhagic shock. Only those animals with inducible nitric oxide synthase activity showed a decreased response to norepinephrine, and this hyporeactivity was reversed with the K_{ATP} channel inhibitor, glibenclamide.**

Conclusions: **The data indicate that profound K_{ATP} activation associated with increased nitric oxide concentrations and inducible nitric oxide synthase induction is a key factor in vascular smooth muscle hyporeactivity characteristic of the late decompensatory phase of hemorrhagic shock in swine.**

TRAUMATIC accidents and hemorrhagic shock continues to be a leading cause of death in patients less than 44 yr of age in the United States and are the fifth leading cause of death in all age groups combined.[1] During hemorrhagic shock, compensatory responses attempt to maintain mean arterial pressure *via* vasoconstriction and extravascular fluid recruitment. The vasoconstriction is primarily mediated through increased concentrations of circulating catecholamines, vasopressin, and activation of the renin-angiotensin system.[2] However, as the shock state continues the compensatory constriction mechanisms fail, resulting in vasodilation, further hypoperfusion, ischemia, and ultimately cellular death and organ failure. A recent publication by Landry and Oliver reviewed the potential mechanisms for vascular smooth muscle hyporeactivity during shock states: decreased concentrations of vasopressin, increased concentrations of nitric oxide, and activation of adenosine triphosphate–sensitive potassium (K_{ATP}) vascular smooth muscle channels.[3] Extensive evidence exists in experimental models of endotoxin shock that the prolonged increased concentrations of nitric oxide cause opening of vascular smooth muscle channels that contributes to the vasodilation and resistance to vasopressors that is observed.[4–6] The increased concentrations of nitric oxide are important in the development of vascular hyporeactivity by causing an increase in cyclic guanosine monophosphate, resulting in activation of myosin light-chain phosphatase and increases in protein G kinase phosphorylation that opens K_{ATP} and K_{CA} channels. The activation of K_{ATP} and K_{CA} channels allows potassium to leave the cell, causing hyperpolarization and subsequently reduced cytoplasmic calcium concentrations.[7,8] Previous work in hemorrhagic shock models showed an association between inducible nitric oxide synthase activity and vascular hyporeactivity in rats, and additional empiric data from swine show *in vivo* vascular decompensation and short-term survival is improved with K_{ATP} channel blockade.[9–13] However, the studies implicating inducible nitric oxide synthase in the development of vascular hyporeactivity have been performed in rats, which may respond differently than other species in regard to the time course of expression and activity of inducible nitric oxide synthase. To further examine the role of constitutive and inducible nitric oxide synthase with K_{ATP} channel activation in decompensated hemorrhagic shock in swine, we evaluated the time course of K_{ATP} channel activation in swine carotid artery vessel rings with nitric oxide concentrations in plasma, liver, lung, and aorta as well as nitric oxide synthase activity in the liver, lung, and aorta.

Materials and Methods

After Institutional Animal Care and Use Committee approval, adolescent Yorkshire swine (30–35 kg, Sus

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scrofa) underwent controlled isobaric arterial hemorrhage with target mean arterial pressures of either 40 mmHg for 2 h (n = 6), 40 mmHg for 4 h (n = 10), or 50 mmHg for 4 h (n = 8). An additional six animals were anesthetized and instrumented but did not undergo controlled arterial hemorrhage. These animals served as controls for the isolated vascular ring experiments (n = 6). All animals were cared for according to the United States Department of Agriculture Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animal Preparation and Monitoring

Anesthesia and Hemodynamic Monitoring Procedures. The swine were fasted overnight with free access to water. In the morning they were sedated with an intramuscular injection of ketamine (250 mg), and anesthetized with halothane by nose cone to facilitate tracheal intubation. During surgical preparation, anesthesia was maintained with halothane (1.0%) while the animals spontaneously ventilated a mixture of oxygen (21%) and nitrogen (78%) through a semiclosed circle system (Narkomed 2B; North American Drager, Telford, PA). The right external jugular vein was isolated, an 8.5-French introducer sheath was inserted, and a continuous thermodilution cardiac output pulmonary artery catheter was advanced through the sheath to measure pulmonary artery pressure and cardiac output (Qvue; Abbott Critical Care, North Chicago, IL). Both femoral arteries were isolated and cannulated with 8.5-French introducer sheaths. A micromanometer (MPC-500; Millar Instruments, Houston, TX) was inserted into the right femoral artery and advanced to the midthoracic aorta for the measurement of mean arterial pressure. The second femoral artery sheath was used for controlled arterial hemorrhage. Physiologic data were displayed on an eight-channel Hewlett-Packard Model 68 clinical monitor (Hewlett-Packard, Andover, MD) and the analog outputs digitized and stored to computer hard disc.

After instrumentation for physiologic monitoring, a right subcostal incision was made and the animals were placed in the right lateral position. After repositioning, the wound edges were infiltrated with bupivacaine (0.5% 15 ml). Plastic wrap was used to restrict the intestines to abdominal cavity and the entire area was covered with plastic wrap to prevent desiccation.

Experimental Protocol

Controlled Arterial Hemorrhage. One hour before the initiation of hemorrhage, the expired halothane concentration was reduced to 0.6% with the animals spontaneously ventilating. Controlled arterial hemorrhage was automated to insure reproducibility. In brief, a customized computer protocol (LABVIEW 5; National Instruments, Austin, TX) monitored the mean arterial pressure. Using a proportional control feedback algorithm, the program controlled the speed and direction of a partial occlusion roller pump (MasterFlex Digital Console Drive; Cole-Parmer Instruments, Chicago, IL) that was connected to a femoral arterial cannula. At the start of the hemorrhage period, the computer program initiated blood withdrawal to decrease the mean arterial pressure to the target pressure over 15 min. During hemorrhage, the blood was stored in a closed reservoir primed with sodium citrate (1.66 g) and porcine heparin (3000 units) to inhibit clot formation. After the initial 15-min rapid hemorrhage, the program maintained the mean arterial pressure at either 40 or 50 mmHg by withdrawal or by reinfusion of the hemorrhage blood as necessary. During the protocol, the volume in the reservoir was gravimetrically measured (Sartorius LA4200; Sartorius Corp, Edgewood, NY) and the data output was stored on computer hard disk with the time stamped mean arterial pressure. During the decompensatory phase of shock, only the blood withdrawn to induce hypotension was administered to maintain the target mean arterial pressure. Global oxygen consumption was calculated using a VMAX 29 metabolic cart (Sensormedics, Yorba Linda, CA). Oxygen debt (ml/kg) during controlled arterial hemorrhage was calculated every minute as the difference between the oxygen consumption (ml/kg/min) after the start of controlled arterial hemorrhage and the oxygen consumption (ml/kg/min) before the start of the protocol. The cumulative oxygen debt was obtained by adding the minute-to-minute oxygen consumption deficits.

Analytical Sampling Procedures

Immediately before hemorrhage and every 30 min thereafter, arterial blood was sampled for measurement of pH, base excess, blood gases, and hemoglobin (IL 1610 Blood Gas Analyzer and 642 Cooximeter; Instrumentation Laboratories, Lexington, KY). Arterial blood samples for serum bicarbonate, lactate, and mixed venous blood obtained for plasma nitrate concentrations were also obtained every 30 min. Lactate was measured with a CMA 600 Analyzer (CMA/Microdialysis, Acton, MA).

Tissue Sampling

At times H_0, H_{60}, and H_{120}, tissue samples were obtained from the liver. Liver tissue was taken from the edge of the left lobe and rapidly immersed in liquid isopentane at −90°C. Bleeding was controlled by application of thrombin, collagen matrix, and direct pressure. After the end of the controlled arterial hemorrhage protocol (H_{120} or H_{240}, depending on the group), carotid arteries were harvested for vessel ring experiments. At the end of the protocol liver, lung, and aortic tissue were obtained and rapidly frozen. The animals were then euthanized by exsanguination. After freezing, all tissue was pulverized with a pestle and mortar under liquid

nitrogen, transferred to chilled cryovials, and stored at −80°C for future analysis.

Determination of Plasma Nitrate

Total plasma nitrate concentrations were determined as an indicator of nitric oxide production. In brief, blood samples were centrifuged (7200*g* for 10 min) at 4°C. The plasma was decanted and stored at −80°C until assayed. Thawed plasma samples were deproteinated by incubating with 100% ethanol for 30 min on ice. The samples were centrifuged (13,000*g* for 10 min), and nitrate concentrations were determined with a Sievers Chemiluminescence Nitric Oxide Analyzer (Sievers 270B NO; Sievers, Boulder, CO). In brief, total nitrates were determined in triplicate by the injection of 10 μl deproteinated plasma into the purge vessel of the Sievers Analyzer containing the reducing agent VCL3 (0.8% in 1 N HCL) at 90°C. During this process plasma nitrite is converted to nitrate, and the total nitrate concentration was calculated by comparing the area under the curve to those generated with standard solutions of sodium nitrate.

Determination of Tissue Nitrate

Frozen pulverized tissue samples were homogenized (1:4 weight/volume) at −4°C in ice-cold homogenization buffer composed of the following (mM): 50 Tris HCL, 0.1 EDTA, 0.1 EGTA, 12 mercaptoethanol, and 100 μg/ml phenylmethylsulfonyl fluoride (pH 7.4). The homogenate was further incubated with diethylthiocarbamate (1 mM) for 10 min and then deproteinated with ice-cold 1% TCA. The samples were centrifuged (13,000*g* for 10 min), and the nitrate concentration was determined using the Sievers 270 B nitric oxide analyzer as described above.[14,15]

Determination of Tissue Nitric Oxide Synthase Activity

Frozen pulverized tissue samples were homogenized (1:4 W/V) at −4°C in ice-cold homogenization buffer (pH 7.4) that consisted of the following: 50 mM HEPES, 100 μM dithiothreitol, 0.55 μM leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin in double distilled water. 300 μl Dowex 50W (8× Na form, 1:1 weight/volume double distilled water pH 7.4) was added to the homogenate to trap the endogenous L-arginine and the homogenate was centrifuged in spin cups (13000*g* for 0.5 min) at 4°C to separate the supernatant from the Dowex. One hundred μl of the supernatant was incubated with 400 μl of reaction buffer at 37°C for 60 min. For total nitric oxide synthase activity the final concentration of reaction mixture was 25 mM Tris HCl, 2.0 mM dithiothreitol, 2.5 u/ml calmodulin, 60 mM L-valine, 5 μM tetrahydrobiopterin, 2 mM FAD, 1.2 mM $MgCl_2$, 1.5 mM NADPH, 2 mM $CaCl_2$ and 1 pmol/μl [^{14}C] L-arginine. The reaction was terminated by the addition of 750 μl cold (4°C) stopping buffer (pH 5.5) 50 mM HEPES, 5.0 mM EDTA, 5.0 mM EGTA. Two hundred μl Dowex 50W-×8 resin (1:1 weight/volume) was added to trap excess [^{14}C] L-arginine, and the reaction mixture was transferred to spin cups and centrifuged (15,000*g* for 30 s). Calcium independent nitric oxide synthase activity was determined by incubating the tissue supernatant in the presence of EGTA and the absence of calcium and calmodulin. The activity of constitutive nitric oxide synthase was calculated as the difference of total and calcium independent nitric oxide synthase activity. All calculations were corrected for background activity, which was determined by performing all steps in the absence of tissue supernatant. All assays were performed in duplicate. The [^{14}C] L-arginine (348 mCi/mmol) was obtained from Amersham Biosciences (Piscataway, NJ).[16]

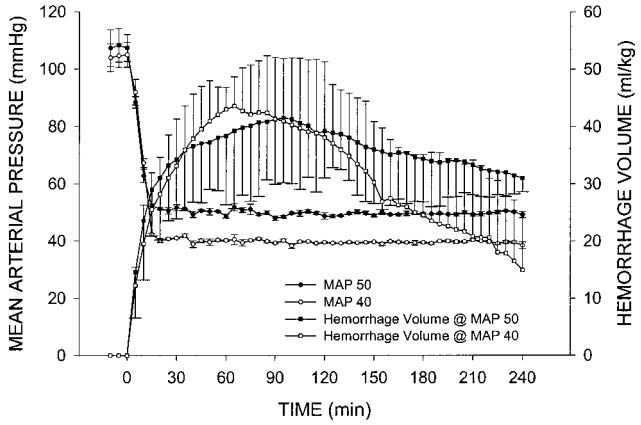

Fig. 1. This figure shows the mean arterial pressure and hemorrhage volume measurements over the course of the experiment for both the mean arterial pressure 40 and mean arterial pressure 50 groups.

Determination of Tissue Inducible Nitric Oxide Synthase Expression

In brief, pulverized frozen tissue samples of liver, lungs, and aorta were lysed in ice-cold buffer consisting (1:10 weight/volume) of 10 mM HEPES (pH 7.4), 38 mM NaCl, 25 mg/ml phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, and 1 mg/ml aprotinin. After vortexing, the samples were placed on ice for 30–45 min. Then after brief sonication, samples were centrifuged (1000*g* for 10 min), and the resulting supernatant was used for Western blotting. Twenty-five μg of cell homogenate was boiled in sample buffer (62.5 mmol Tris HCl (pH 6.8), 2% SDS, 10% glycerol, 5% mercaptoethanol, and 0.005% bromophenol blue) for 2 min and subjected to 10% SDS-polyacrylamide gel electrophoresis using mini-gel equipment (Bio-Rad Laboratories, Hercules, CA). For detection of inducible nitric oxide synthase, the membranes were incubated with an inducible nitric oxide synthase specific primary rabbit polyclonal antibody (1:500) (Transduction Laboratories, San Diego, CA). The

Table 1. Laboratory and Oxygen Consumption Measurements

Variable	Group	Time (min) H_0	H_{30}	H_{60}	H_{90}
Animals (No.)	MAP 50	8	8	8	8
	MAP 40	16	16	16	16
Lactate (mM)	MAP 50	1.4 ± 0.4	2.6 ± 1.2*	4.1 ± 2.1*	6.3 ± 2.4*
	MAP 40	1.3 ± 0.3	3.8 ± 1.6*	7.4 ± 3.0*†	11.3 ± 3.1*†
pH	MAP 50	7.45 ± 0.02	7.44 ± 0.04	7.43 ± 0.05	7.43 ± 0.03
	MAP 40	7.43 ± 0.03	7.44 ± 0.05	7.42 ± 0.06	7.32 ± 0.12
Base Excess (meq/dl)	MAP 50	4.8 ± 1.6	3.8 ± 2.1*	1.9 ± 2.1*	0.1 ± 2.6*
	MAP 40	4.2 ± 1.6	1.3 ± 2.1*†	−4.3 ± 3.5*†	−9.4 ± 5.7*†
Bicarbonate (meq/dl)	MAP 50	31.2 ± 1.5	30.1 ± 1.3	28.5 ± 2.1*	26.7 ± 2.6*
	MAP 40	30.9 ± 1.6	27.9 ± 1.3*	23.8 ± 5.1*†	19.7 ± 5.2*†
VO_2 (ml/kg/min)	MAP 50	5.6 ± 0.4	4.8 ± 0.4*	4.3 ± 0.5*	4.2 ± 0.8*
	MAP 40	5.8 ± 0.6	4.3 ± 0.6*†	4.0 ± 0.9*†	3.6 ± 0.9*†
O_2 Debt (ml/kg)	MAP 50	0.0 ± 0.0	21.8 ± 1.7*	55.5 ± 3.5*	97.3 ± 8.3*
	MAP 40	0.0 ± 0.0	28.7 ± 2.5*	75.5 ± 5.9*†	136.5 ± 9.7*†

Data are presented as mean with SD. H_0 measurements were made immediately before the start of the controlled arterial hemorrhage protocol.

MAP = mean arterial pressure; VO_2 = oxygen consumption.

* $P < 0.05$ compared with the H_0 sample data in the same group; † $P < 0.05$ compared with matched time sample data between groups.

membranes were then incubated with secondary goat antibody directed towards the antirabbit immunoglobulin G (1:2000). Negative controls were performed by omitting primary antibody. Equal amounts of protein were loaded into each lane and confirmed by Ponceau reagent (Sigma-Aldrich, St. Louis, MO). Chemiluminescence was detected by autoradiography and the integrated optical density of the protein bands was measured using the Scion image program.

Isolated Carotid Ring Experimental Protocols

At the end of either 120 or 240 min of hemorrhage, the carotid arteries were excised and placed in cold Krebs bicarbonate buffer (pH 7.4) of the following composition (mM): 119 NaCl, 25 $NaHCO_3$, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, and 5.6 glucose. Within 30 min, the vessels were cleaned and 3–4 mm rings with intact endothelium were suspended in organ chambers filled with aerated Krebs-Ringer solution (95% O_2-5% CO_2). Four to six carotid artery rings with intact endothelium were studied simultaneously from each pig. Individual rings were placed between wire holders, and each upper holder was attached to a force transducer (FT-03; Grass, Quincy, MA) for continuous recording of isometric tension (RS 3400; Gould, Cleveland, OH). The lower holder was suspended from a hook in a Krebs-filled (25 ml) water-jacketed organ chamber maintained at 37°C and aerated with a mixture of 95% O_2-5% CO_2. The rings were equilibrated for 60 min at their optimal length tension relationship (4–5 g, preliminary experiments). After an additional 3–4 g precontraction of the rings with KCl, the presence of endothelium was verified with acetylcholine (10^{-7} M) by at least 75% relaxation in rings. All rings were equilibrated (4–5 g tension) for an additional 45 min. After equilibration, cumulative concentration curves to norepinephrine were obtained in

Table 2. Physiologic Measurements

Variable	Group	Time (min) H_0	H_{30}	H_{60}	H_{90}
Animals (No.)	MAP 50	8	8	8	8
	MAP 40	16	16	16	16
MAP (mmHg)	MAP 50	104.5 ± 4.9	50.3 ± 0.7*	49.8 ± 0.7*	49.8 ± 0.5*
	MAP 40	106.2 ± 8.8	40.1 ± 0.4*†	40.3 ± 0.5*†	39.3 ± 1.5*†
Heart Rate (beats/min)	MAP 50	144.3 ± 20.2	218.3 ± 18.1*	223.6 ± 13.3*	237.5 ± 16.2*
	MAP 40	146.3 ± 11.1	212.6 ± 42.1*	247.0 ± 8.3*†	246.9 ± 14.2*
CVP (mmHg)	MAP 50	3.0 ± 1.7	−2.6 ± 1.4*	−3.0 ± 1.5*	−3.5 ± 1.2*
	MAP 40	2.1 ± 1.6	−5.4 ± 1.3*†	−4.4 ± 2.5*	−4.3 ± 2.2*
Cardiac Index (ml/kg/min)	MAP 50	158.4 ± 21.3	90.0 ± 17.7*	76.4 ± 21.8*	70.0 ± 23.5*
	MAP 40	161.4 ± 25.2	68.7 ± 19.1*†	49.0 ± 15.5*†	47.8 ± 17.3*†
SVR (dyne-cm/sec^{-5})	MAP 50	1142 ± 193	1526 ± 335*	1776 ± 631*	1977 ± 621*
	MAP 40	1208 ± 248	1656 ± 445*	2246 ± 941*	2341 ± 648*

Data are presented as mean with SD. The initial hemodynamic measurements were made before the start of the controlled arterial hemorrhage.

CVP = central venous pressure; MAP = mean arterial pressure; SVR = systemic vascular resistance.

* $P < 0.05$ compared with the H_0 sample data in the same group; † $P < 0.05$ compared with matched time sample data between groups.

Table 1. *Continued*

Time (min)				
H_{120}	H_{150}	H_{180}	H_{210}	H_{240}
8	8	8	8	8
16	10	10	10	10
7.3 ± 3.7*	7.8 ± 3.3*	8.1 ± 3.5*	8.4 ± 3.6*	8.2 ± 3.2*
16.6 ± 2.9*†	16.7 ± 3.3*†	16.4 ± 2.6*†	16.3 ± 2.4*†	16.1 ± 2.2*†
7.42 ± 0.03*	7.39 ± 0.04*	7.40 ± 0.05*	7.36 ± 0.05*	7.37 ± 0.07*
7.27 ± 0.13*†	7.21 ± 0.08*†	7.21 ± 0.07*†	7.25 ± 0.05*†	7.24 ± 0.11*†
−0.9 ± 2.3*	−2.3 ± 1.1*	−2.7 ± 1.2*	−3.9 ± 1.4*	−4.1 ± 1.7*
−12.3 ± 5.6*†	−13.0 ± 3.6*†	−13.7 ± 3.7*†	−12.6 ± 4.5*†	−12.2 ± 6.9*†
25.7 ± 3.6*	24.6 ± 3.1*	24.2 ± 3.4*	23.6 ± 2.9*	21.6 ± 3.2*
16.7 ± 5.8*†	12.7 ± 4.7	13.1 ± 3.7*†	14.2 ± 5.1*†	14.1 ± 5.4*†
4.1 ± 0.4*	4.2 ± 0.5*	4.3 ± 0.5*	4.2 ± 0.4*	4.4 ± 0.5*
3.5 ± 0.8*†	3.6 ± 0.5*†	3.8 ± 0.5*†	4.1 ± 0.4*†	3.9 ± 0.2*†
140.2 ± 13.2*	187.1 ± 15.6*	235.2 ± 18.9*	279.1 ± 21.4*	324.3 ± 23.4*
209.6 ± 13.0*†	286.8 ± 26.7*†	338.8 ± 30.9*†	384.4 ± 37.5*†	428.6 ± 52.8*†

rings incubated for 15 min with the K_{ATP} channel inhibitor glibenclamide (10 mM) or with the vehicle dimethylsulfoxide, which has no influence on tension level.[5,17] At the end of the protocol, the functional integrity of the endothelium was again documented by acetylcholine (10^{-7})-induced relaxation.

Statistical Analysis

Data are presented as mean with SD. Differences between groups for nonrecurring measurements were assessed using analysis of variance. Differences between and within groups over the course of the experiment were determined by analysis of variance between groups for the dependent variable (*i.e.*, lactate, oxygen consumption) with repeated measures over time. Within and between group testing was accompanied by a Tukey honest significant difference multiple range test to correct for multiple comparisons. Values were considered statistically different when $P < 0.05$ after correction for multiple comparisons.

Results

Hemorrhage, Hemodynamic and Metabolic Measurements

Figure 1 shows the accuracy and precision of the automated hemorrhage system for the induction and maintenance of target mean arterial pressure at 40 mmHg or 50 mmHg and the accumulation of the hemorrhage volume (ml/kg). The peak hemorrhage volume was 44.1 ± 9.4 ml/kg at 75 ± 17 min in the mean arterial pressure 40 group and 34.1 ± 4.8 ml/kg at 102 ± 16 min ($P < 0.05$) in the mean arterial pressure 50 group. Despite the lower target pressure and the higher peak hemorrhage volume, the mean arterial pressure 40 group required a significantly larger amount of reinfusion of the hemorrhage volume to maintain the target pressure and at H_{240}. The mean arterial pressure 40 group had a significantly lower residual hemorrhage volume of 14.9 ± 9.5 *versus*. 25.6 ± 1.8 ml/kg ($P < 0.05$) in the mean arterial pressure 50 group.

Measurements of oxygen consumption were recorded

Table 2. *Continued*

Time (min)				
H_{120}	H_{150}	H_{180}	H_{210}	H_{240}
8	8	8	8	8
16	10	10	10	10
49.9 ± 0.4*	49.9 ± 0.4*	49.9 ± 0.4*	50.0 ± 0.7*	50.0 ± 0.7*
39.9 ± 0.4*†	39.8 ± 0.4*†	39.8 ± 0.4*†	39.8 ± 0.4*†	39.2 ± 1.7*†
235.6 ± 8.5*	235.5 ± 12.2*	235.0 ± 19.4*	233.0 ± 13.8*	229.4 ± 16.1*
229.9 ± 28.2*	221.2 ± 26.1*	214.3 ± 25.7*	214.0 ± 30.2*	221.0 ± 26.5*
−3.6 ± 1.1*	−3.6 ± 1.1*	−3.3 ± 1.3*	−2.5 ± 1.4*	−2.1 ± 1.6*
−4.1 ± 5.1*	−2.7 ± 2.9*	−2.3 ± 3.1*	−2.0 ± 4.0*	−0.8 ± 2.1*
63.9 ± 17.2*	62.3 ± 12.9*	61.8 ± 12.8*	63.0 ± 11.9*	61.7 ± 13.3*
44.8 ± 11.8*†	47.3 ± 11.6*†	51.2 ± 12.9*	52.3 ± 11.5*	55.0 ± 13.2*
2146 ± 679*	2151 ± 580*	2163 ± 514*	2100 ± 433*	2140 ± 489*
2825 ± 745*†	2343 ± 361*	2063 ± 519*	1923 ± 674*	1799 ± 442*

and metabolic changes (pH, serum bicarbonate, base deficit, and lactic acid) followed through serial blood samples (table 1). The animals in the mean arterial pressure 50 group tolerated the controlled hemorrhage better than the animals in the mean arterial pressure 40 group based on the parameters measured. The oxygen consumption in the mean arterial pressure 50 animals showed less of a decrement than the mean arterial pressure 40 animals and therefore they accumulated less of an oxygen debt. The trends in the measurements of clinically acceptable end points of resuscitation (*i.e.*, base excess and lactate concentration) further exemplify this point. Lactate and base deficit accumulated quicker and to a substantially higher concentration in the more severely hemorrhaged animals reaching statistical significance ($P < 0.05$) at 60 min for lactate and in 30 min for base deficit. These changes were also reflected in significant differences ($P < 0.05$) between groups in pH beginning at time H_{120} and in serum bicarbonate concentrations starting at time H_{60}.

Hemodynamic measurements are shown in table 2. Overall, the hemodynamics are consistent with the parameters expected in hemorrhagic shock. There were variable isolated statistically significant between group differences in all measured variables; however, mean arterial pressure and cardiac index were the only parameters with sustained statistically different measurements. The difference in mean arterial pressure was obviously protocol driven. The cardiac index decreased substantially quicker and to a lower level in the more severely hemorrhaged animals. There was a statistically significant difference from 30 min through 150 min. During the remainder of the experiment, the animals in the mean arterial pressure 40 group still had a lower (although not statistically different) cardiac index.

Blood and Tissue Nitrate and Nitric Oxide Synthase Analysis

Samples were taken from all animals for blood analysis every 30 min and liver analysis every 60 min. Lung and aorta samples were obtained at H_{120} (n = 6, mean arterial pressure 40) and at H_{240} (n = 10 mean arterial pressure 40, n = 8 mean arterial pressure 50) depending on when the animals were euthanized. Both the blood and liver nitrate concentrations increased from baseline throughout the protocol in both the mean arterial pressure 40 and 50 groups ($P < 0.05$) (table 3). However, between groups the blood nitrate concentrations were significantly higher in the mean arterial pressure 40 group at all times during the hemorrhage protocol ($P < 0.05$). Although the liver nitrate concentrations also increased throughout the protocol, the only differences between the mean arterial pressure 40 and 50 groups occurred at H_{240} (3.7 ± 1.8 and 2.3 ± 1.1 μmol/μg dry weight, respectively, $P < 0.05$). In addition, lung and aorta nitrate concentrations for the mean arterial pressure 40 animals was also significantly higher than the mean arterial pressure 50 group at H_{240} ($P < 0.05$).

Nitric oxide synthase activity was also measured for both total nitric oxide synthase and the inducible nitric oxide synthase isoform (fig. 2). The activity of the constitutive nitric oxide synthase was calculated as the difference between the total nitric oxide synthase and inducible nitric oxide synthase activity. Activities were measured in liver tissue obtained at H_0, H_{60}, H_{120}, and H_{240} and in lung and aorta samples obtained at H_{120} and H_{240}. The total nitric oxide synthase activity in the liver rose steadily in the mean arterial pressure 40 group and the increase in total nitric oxide synthase activity was significantly higher at all time points when compared to the mean arterial pressure 50 group (fig. 2 A, $P < 0.05$). At H_{60} and H_{120}, constitutive nitric oxide synthase accounted for the majority (96–97%) of the total nitric oxide synthase activity for both groups. However, at H_{240}, inducible nitric oxide synthase activity represented 26% of the total nitric oxide synthase activity in the mean arterial pressure 40 group with minimal activity in the mean arterial pressure 50 group ($P < 0.05$). The data from lung tissue were similar to liver nitric oxide synthase data, with the mean arterial pressure 40 group attaining significantly higher peaks with larger inducible nitric oxide synthase (4 *versus* 27%, $P < 0.05$) compared with the mean arterial pressure 50 group. Although the aorta tissue showed significant differences in total nitric oxide synthase activity between the two groups, there was no evidence of increased inducible nitric oxide synthase activity in the aorta at H_{240} (fig. 2 C). Compared to the optical density of negative controls, western blots of tissue samples from aorta, liver, and lung tissues harvested at H_{120} from the mean arterial pressure 40 group and at H_{240} for the mean arterial pressure 50 group showed no evidence of inducible nitric oxide synthase expression. However, inducible nitric oxide synthase protein in the liver and lung tissue blots from the mean arterial pressure 40 group at H_{240} was significantly increased compared to the negative controls (optical density, 218.3 ± 40.8 and 97.3 ± 7.6 *versus* 11.7 ± 4.1, respectively). The optical density of the aorta tissue for all groups was not different from the negative controls. These differences in the optical density indicate an absence of inducible nitric oxide synthase expression in the mean arterial pressure 50 group and the presence of inducible nitric oxide synthase protein in the mean arterial pressure 40 group only in the liver and lung at H_{240} and not H_{120}.

Isolate Carotid Artery Vessel Ring Experiments

Isolated carotid artery rings with intact endothelium from animals in the mean arterial pressure 40 group sacrificed at H_{120} (n = 6) showed no difference in their response to norepinephrine compared to the instrumented time control animals (fig. 3). The remaining

Table 3. Blood and Tissue Nitrate Measurements

Variable	Group	Time (min) H_0	H_{60}	H_{120}	H_{180}	H_{210}	H_{240}
NO_x liver (μmol/μg dry weight)	MAP 50	1.3 ± 1.7 (n = 8)	1.9 ± 1.4 (n = 8)	2.2 ± 1.6* (n = 8)	NM	NM	2.3 ± 1.1* (n = 8)
	MAP 40	1.5 ± 1.7 (n = 16)	2.3 ± 2.6* (n = 16)	2.5 ± 1.8* (n = 16)	NM	NM	3.7 ± 1.8*‡† (n = 10)
NO_x lung (μmol/μg dry weight)	MAP 50	NM	NM	NM	NM	NM	2.7 ± 1.2 (n = 8)
	MAP 40	NM	NM	3.4 ± 1.1 (n = 6)	NM	NM	4.2 ± 2.1‡† (n = 10)
NO_x aorta (μmol/μg dry weight)	MAP 50	NM	NM	NM	NM	NM	2.1 ± 1.8 (n = 8)
	MAP 40	NM	NM	2.6 ± 1.2 (n = 6)	NM	NM	3.2 ± 1.9† (n = 10)
NO_x plasma (μM)	MAP 50	5.1 ± 0.6 (n = 8)	6.8 ± 0.6* (n = 8)	7.5 ± 1.2* (n = 8)	8.6 ± 0.6* (n = 8)	8.7 ± 0.5* (n = 8)	9.2 ± 0.7* (n = 8)
	MAP 40	4.9 ± 0.7 (n = 16)	8.2 ± 0.4*† (n = 16)	9.5 ± 1.0*† (n = 16)	10.2 ± 0.9*† (n = 10)	11.2 ± 1.0*† (n = 10)	13.9 ± 1.2*† (n = 10)

Data are presented as mean with standard deviation. H_0 measurements were made on samples collected immediately before the start of the controlled arterial hemorrhage protocol.

MAP = mean arterial pressure; NM = not measured; NO = nitric oxide.

* $P < 0.05$ compared with the H_0 sample data in the same group. † $P < 0.05$ compared with matched time sample data between groups. ‡ $P < 0.05$ tissue sample data at H_{240} compared with tissue sample data at H_{120}.

vessel rings were obtained from the animals sacrificed at H_{240}. The rings from the mean arterial pressure 50 group showed the same response to norepinephrine as the control group and the mean arterial pressure 40 animals at H_{120}. However, the vessel rings from the mean arterial pressure 40 group at H_{240} had significantly lower response to norepinephrine with approximately half the contractile force of the control group. However, after the rings were incubated with glibenclamide, a reversible K_{ATP} channel inhibitor, they showed a recovery of responsiveness to norepinephrine. Starting at 10^{-7} M norepinephrine through 10^{-5} M norepinephrine the vessels showed signs of increased responsiveness but still had not regained normal responsiveness. At higher doses of norepinephrine, 10^{-5} M norepinephrine through 10^{-4} M, the vessels reacted the same as the control group.

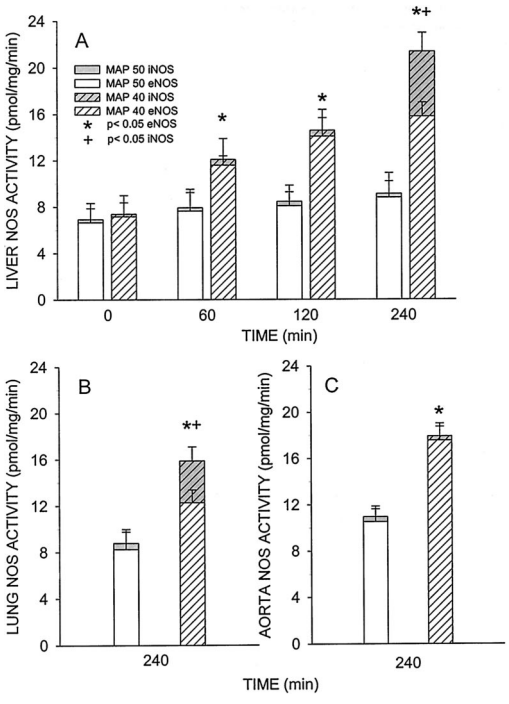

Fig. 2. Tissue nitric oxide synthase (NOS) activity. Panel A shows total nitric oxide synthase activity for the liver with the contribution from the constitutive nitric oxide synthase (cNOS) and inducible nitric oxide synthase (iNOS) forms. Panels B and C show the total nitric oxide synthase activity from lung and aorta tissue after 240 min of hemorrhagic shock.

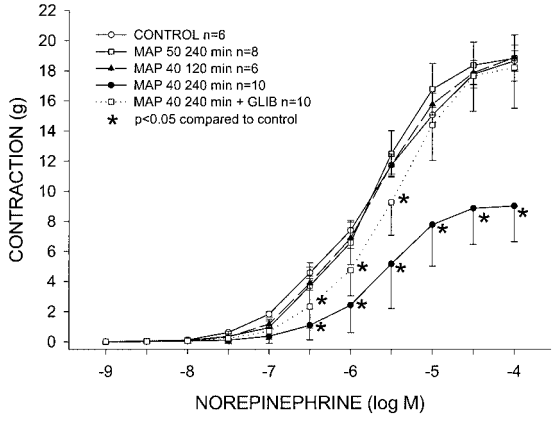

Fig. 3. Isolated carotid artery vessel ring K_{ATP} channel activation. This figure shows the force of contraction (grams) *versus* the cumulative norepinephrine dose.

Discussion

As observed by Wiggers in the 1940s, prolonged controlled hemorrhagic shock eventually results in a decompensatory phase of shock characterized by an inability to maintain mean arterial pressure without volume infusion.[18] In our model of controlled arterial hemorrhage, we have observed both in the current and previous experiments that the major cardiovascular pertubation during the decompensatory phase of shock is mediated by the dilation of the vascular system and not by failure of myocardial contractility.[19] This is shown in the mean arterial pressure 50 group, in which the mean arterial pressure, systemic vascular resistance, and cardiac index remained essentially unchanged after the onset of decompensation despite the return of some of the hemorrhage volume. However, in the mean arterial pressure 40 group, the cardiac index increased with a more pronounced hemorrhage volume return secondary to a decreasing systemic vascular resistance noted during the decompensated shock phase. The decreasing systemic vascular resistance is a manifestation of peripheral vasodilation. Furthermore, the data from this study indicate that the vasodilation is associated with 1) progressively higher concentrations of nitric oxide in blood and tissue samples as indicted by nitrate concentrations, 2) the presence of inducible nitric oxide synthase activity after 4 h of shock in mean arterial pressure 40 group, and 3) *in vitro* glibenclamide reversible K_{ATP} channel activation that occurred only in the mean arterial pressure 40 group at 4 h of shock.

The automated isobaric controlled arterial hemorrhage model was chosen from the numerous methods to induce hemorrhagic shock ("uncontrolled bleeding" or "controlled prefixed volume") because of the reliability to induce a prolonged, measurable state of decompensatory shock. The computer system uses a controlled proportional feedback algorithm that continuously measures the difference between target and actual mean arterial pressure. By automatically adjusting the speed and direction of the roller pump, the system is able to provide rapid and precise changes in the blood volume and mean arterial pressure as illustrated in figure 1 with minimal researcher intervention. Using this method, the peak hemorrhage volume represents a transition from compensatory to decompensatory shock since the animals were no longer able to maintain their own blood pressure through intrinsic homeostatic mechanisms without the assistance of volume reinfusion. In both animal groups (mean arterial pressure 40 and mean arterial pressure 50), lactate concentrations continue to increase and base deficit decrease after the onset of the decompensatory phase of shock ($\approx H_{90}$). After approximately another hour, the lactate and base deficit concentrations stabilized representing equilibrium between aerobic and anaerobic metabolism despite the fact that the oxygen consumption remained low and oxygen debt continued to accumulate.

Nitric Oxide and Nitric Oxide Synthase Activity during Hemorrhagic Shock

First described as endothelium-derived relaxing factor, nitric oxide is a powerful vasodilator that is synthesized from the guanidine-group of L-arginine by the nitric oxide synthase; a group of enzymes consisting of a calcium-dependent constitutive and a calcium-independent inducible form. *In vivo*, constitutive nitric oxide located in the vascular endothelium plays a fundamental role in the maintenance of vasomotor tone and local blood flow. Although the underlying molecular mechanisms remain unclear, the increase in shear stress secondary to the changes in blood flow that occur during hemorrhagic shock is a major factor in the increase in constitutive nitric oxide synthase activity in the vascular endothelium. In addition, β-adrenergic effects from endogenous catecholamines also have a positive effect on nitric oxide production. However, a complete understanding of the complex interaction of local mediators of vascular tone and nitric oxide pathways that regulate arterial pressure is still not well defined.[20–22] Inducible nitric oxide, on the other hand, is not normally present in tissues unless factors such as tumor necrosis factor alpha and or lipopolysaccharide signal the production of messenger RNA and subsequent protein synthesis.

In rats, there is substantial evidence of increased nitric oxide concentrations from constitutive and inducible nitric oxide synthase are during hemorrhagic shock and that it contributes to deleterious effects during severe shock states.[9,10,23–27] However, previous studies of swine during shock have not provided convincing direct measurements of nitric oxide and inducible nitric oxide synthase activity in relation to selective nitric oxide synthase blockade or improvement in vascular function.[12,28,29] In fact, previous studies of septic shock and ischemia reperfusion in swine indicate that direct and indirect evidence for inducible nitric oxide synthase activity could not directly implicate the inducible nitric oxide synthase in the generation of deleterious outcomes.[28–31] Although studies have looked at the relationship of nitric oxide synthase activity during septic shock, only one previous rat study of hemorrhagic shock provides direct measurement of changes in nitric oxide synthase activity in relation to vascular hyporeactivity.[9] In that study, rats were subjected to severe hemorrhage for either 150 or 330 min and Thiemermann *et al.* found that there was an increase in inducible nitric oxide synthase activity in the liver and lung, but not the aorta, at 150 min. However, all three tissues showed evidence of inducible nitric oxide synthase activation at 330 min. In our study, nitric oxide synthase activity in the liver, lung, and aorta was measured at 120 and 240 min. In contrast to Thiemermann *et al.*'s study in rats, the in-

crease in nitric oxide synthase activity in all the swine tissues at 120 min was attributable only to increased constitutive nitric oxide synthase activity. In addition, in the swine inducible nitric oxide synthase activity was present in liver and lung tissue, but not aorta, at 240 min of severe hemorrhage. Thus, in the current experiment the increased nitrate observed in the blood and liver appears to be mediated primarily by constitutive nitric oxide synthase for 120 min. In addition, constitutive nitric oxide synthase is probably responsible for the majority of nitric oxide production for as long as 240 min because the relative activity of the inducible nitric oxide synthase was a minor proportion of the total activity. Thus, although Thiemermann *et al.*'s study and our study demonstrate an increase in inducible nitric oxide synthase in late hemorrhagic shock, we did not find any evidence for inducible nitric oxide synthase activity at 120 min, which may be explained by a difference in time course for the rats and swine. As the controlled arterial hemorrhage was terminated at 240 min in our protocol, we were unable to determine when the inducible nitric oxide synthase activity would have become the major source of nitrate concentrations in the various tissues and blood.

Ultimately, the difference between nitric oxide derived from constitutive nitric oxide synthase and inducible nitric oxide synthase is the magnitude of production. Constitutive nitric oxide synthase produces nitric oxide in the picomolar concentration range whereas inducible nitric oxide synthase produces nitric oxide in the nanomolar range.[22] This is in relation to their respective functions, with constitutive nitric oxide synthase primarily being responsible for basal tone in vascular smooth muscle; inducible nitric oxide synthase assists in immunologic function. However, high concentrations of nitric oxide from either source can suppress mitochondrial respiration,[32] impair the vascular response to catecholamines and nitrosylate proteins, form peroxynitrite, and induce cellular death.[24,31,33–35] Although we saw progressively higher concentrations of NOx in all tissues sampled, we were unable to separate the relative contributions from constitutive nitric oxide synthase and inducible nitric oxide synthase in the final NOx concentrations. Additional *in vivo* studies, which include specific constitutive nitric oxide synthase and inducible nitric oxide synthase inhibitors, would be needed to determine those proportions.

K_{ATP} Channel Activation

There is growing indirect evidence that K_{ATP} channel activation plays a major role in pathologic vasodilation that occurs during hemorrhagic shock.[11–13] There is also evidence to support the hypothesis that high concentrations of nitric oxide are associated with profound activation of K_{ATP} channel activation in isolated vessels from dogs, rabbits, and swine.[4,36,37] In our study, we harvested carotid arteries from the swine and performed vessel ring contractions with and without exposure to glibenclamide. There was no difference in norepinephrine response between the vessels from control animals and the mean arterial pressure 50 group. However, the vessels from the animals subjected to controlled arterial hemorrhage at a mean arterial pressure of 40 mmHg that were not exposed to glibenclamide showed normal reactivity to norepinephrine at 120 min and a marked decrease in response after 240 min. Reactivity of the paired rings incubated with glibenclamide was improved at intermediate concentrations of norepinephrine and normalized at the higher concentration of norepinephrine. These data show that although decompensation started in all the animals between 90 and 120 min, only the animals subjected to a mean arterial pressure of 40 mmHg for 240 min had significantly increased NOx tissue concentrations and evidence of inducible nitric oxide synthase activation. This is the first report in swine that links increased NOx tissue concentrations and inducible nitric oxide synthase activation to profound K_{ATP} channel activation. Although Thiemermann *et al.* showed profound vessel hyporeactivity in rats that displayed aortic inducible nitric oxide synthase activity, they did not provide evidence to the mechanism of the dysfunction.[9] Although the time course may be different, these data suggest that K_{ATP} channel activation plays a significant role in the late decompensatory phase of shock.

Still, other unmeasured factors may play a role in vascular hyporeactivity that occurs during hemorrhagic shock. Other mediators, such as adenosine and calcitonin gene-related peptide, have a deleterious impact on K_{ATP} channel activation.[7,8] In addition, other ion channels and receptors may play a contributory role in the vasodilation and interpretation of the data presented. For example, K_{CA} channels are also abundantly expressed in vascular smooth muscle, and their open state probability is enhanced by nitric oxide mediated increases in cyclic guanosine monophosphate and protein G kinase phosphorylation.[38] Another point to note is the effect of β-adrenoreceptors on vascular smooth muscle tone. β-adrenoreceptors are located primarily on endothelial cells and mediate relaxation through nitric oxide synthase pathways with increases in cyclic guanosine monophosphate.[39] Although the *in vivo* impact cannot be determined from our experiments, our *in vitro* ring experiments were conducted in endothelium-denuded vascular rings, which significantly decreases any contribution of β-adrenoreceptors to vascular relaxation. Still, these potential mechanisms must be considered in the interpretation of the data. Thus, the inability of the glibenclamide-treated vessels to normalize at the lower concentrations of norepinephrine may be explained by further experiments conducted with specific blockers of K_{CA} channels and β-adrenoreceptors.

In conclusion, our results obtained during different degrees and duration of hemorrhagic shock in swine indicate that nitric oxide concentrations increase during hemorrhagic shock. At 4 h there was a statistically significant increase in inducible nitric oxide synthase expression compared with baseline values, and the time course for inducible nitric oxide synthase activation appears to be longer than that reported in rats.[22] In those animals who were hemorrhaged to 40 mmHg for 4 h, there is evidence of K_{ATP} channel activation that was probably mediated by the rising concentrations of nitric oxide. As with the abundance of evidence in experimental septic shock, these findings may help to explain hypotension resilient to vasopressors that is associated with the late decompensatory phase of hemorrhagic shock. Further work needs to be done to fully access the *in vivo* role of nitric oxide in K_{ATP} channel activation, especially in relation to other receptors and ion channels that have a role in vascular smooth muscle tone.

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